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Analysis of mucosal bladder leucocyte subpopulations in patients treated with intravesical Bacillus Calmette-Guerin

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Summary. Immunohistochemical techniques were used to investigate leucocyte subpopulations in the bladders of patients with superficial transitional cell carcinoma treated with BCG Pasteur. Leucocyte subsets were enumerated with a panel of monoclonal antibodies which included CD3, CD4, CD8, TQ1,Leu7, CD15, HLA-DR, CD25, CD22. We demonstrated in the bladders of patients treated with BCG a particular lymphocyte population; the major subset was an inducer ($CD4^+$, $TQ1^-$) which was activated ($CD25^+$, $HLA\text{-}DR^+$) and associated with polymorphonuclear eosinophils. There was neither inducer of suppression nor major cytotoxic/suppressive subsets. $CD8^+$ and NK cells could not be the primary mediators of BCG activity. These data supported the hypothesis of a helper T lymphocyte activity associated with lymphokine production and activation of effector killer cells.

Key words: Carcinoma of the bladder – BCG therapy – Immunohistochemical studies – Leucocyte subpopulations – Helper T lymphocyte

Introduction

Since Moralès et al. first reported in 1976 the effectiveness of intravesical BCG in preventing the recurrence of Transitional Cell Carcinoma (TCC) of the bladder, many studies have confirmed these results [3, 7, 9, 13] and indeed instillation of BCG is extensively used for the treatment of superficial TCC, i.e. stage Tis, Ta and T1 (UICC classification). The precise cell mechanism involved in the anti-tumor activity of BCG is always questionnable. However it has long been known that after BCG the bladder mucosa contains mononuclear cells [8], contrary to the polymorphonuclear cell infiltration encountered after intravesical chemotherapy like thiotepa [4]. This fact suggests that anti-tumor activity of BCG is not due to a direct cytotoxic effect nor to an acute inflammatory phenomenon but is mediated by an immune response. Recent investigations based on experiments with animals have produced further evidence which indicate an immunological mechanism; in particular, it has been proved that the response to the BCG is mediated by a T-cell infiltrate [11]. In addition, interleukin 2 (IL2), a lymphokine produced by activated T-lymphocytes, has been demonstrated to be dramatically elevated in the urine of patients treated with intravesical BCG [1, 5]. However, the phenotype of leukocytic subpopulation in the human bladder after BCG therapy has not yet been determined. We, therefore, carried out a prospective biopsy study on bladder mucosa in order to define the composition of the leucocytic cell series which are present in the bladder with or without BCG therapy. The object of this study was to support the preponderance of a T-cell mediated mechanism in anti-tumor activity of BCG in human bladder TCC, and to attempt to define which cells have a killer activity on carcinoma cells.

Material and methods

Tissues

Between January 1987 and January 1988 frozen tissue from mucosa of the bladder was selected from 41 patients. 3 of these patients have had the benefit of several consecutive biopsies (2 had 2 and 1 had 3) so a total of 45 biopsy specimens were examined. These were separated into three groups.

- *Group 1*
27 specimens from 26 patients with superficial TCC (2 Tis, 19 Ta, 6 T1) treated by intravesical instillation of BCG.

- *Group 2*
13 specimens from 12 patients with TCC (7 Ta and T1, 6 T2 and T3) without any BCG. 2 patients with Ta and T1 TCC respectively had bladder biopsies prior to and after BCG therapy. The former specimens were integrated into group 2 and the latter into group 1:

Table 1. Antibodies

Antibody/CD	Source	Specificity	Dilution
Leu4/CD3	Becton Dickinson	all T-lymphocytes	1/100
Leu2a/CD8	Becton Dickinson	suppressor/cytotoxic T-cells	1/100
Leu3a/CD4	Becton Dickinson	helper/inducer T-cells, macrophages	1/100
TQ1/NC	Coulter	T, B and null cells. Within $CD4^+$ subset, $CD4^+$ TQ1 - population contains the majority of helper function	1/5
Leu7/NC	Becton Dickinson	Human Natural Killer-1	1/50
OKM-1/CD11	Ortho	granulocytes, monocytes, Human Natural Killer-1	1/100
HLADR/NC	Dakopatts	Histocompatibility class II antigens - all B-cells, activated T-cells	1/100
Anti-Tac/CD25	Dakopatts	receptor of interleukin 2, activated T-cells	1/100
Pan B/CD22	Dakopatts	all B-lymphocytes	1/100

CD = clusters of differentiation; NC = not yet clustered

- *Group 3*

5 specimens from 5 patients without any tumor of the bladder but with cystitis (2), prostatic adenomyoma (2) and an ureteral disease (1).

In Group 1 all the patients were treated with "Pasteur Strain BCG" (120 mg in 100 cc saline intravesically) which was retained in the bladder one and a half hours before voiding; weekly instillations were performed for a total of 6 treatments, followed by one instillation per month for 6 months. Patients were checked by means of control cystoscopy and biopsies. The specimens were removed at the control cystoscopic examination from cystoscopically normal mucosa in order to study the infiltrate determined by the BCG and not the tumor-linked infiltrate. In every group, tissue obtained by cystoscopy was immediately snap-frozen in liquid nitrogen. Then multiple serial sections were performed. One frozen section was stained with hematoxylin plus eosin to appraise the quality of the sample. Tussue blocks for routine histological examination were also performed in all cases after 10% formalin fixation.

Immunocytochemistry

The antibodies used, their sources and specificity are listed in Table 1. Cryostat sections, 5 μm in thickness, were air-dried (minimal 2H at romm temperature) and fixed in acetone for 10 mn, then labelled with a three-step indirect immunoperoxidase technique. The sections were incubated sequentially with a primary antibody appropriately diluted, a sheep anti-mouse peroxidase conjugated antibody, and a rabbit anti-sheep peroxidase conjugated antibody (Dakopatts). Incubations were for 30 min at room temperature, followed by extensive washing. Peroxidase activity was shown with the use of 3'.5' diaminobenzidine (0.6 mg/ml) and hydrogen peroxide (0.01%) at room temperature. Nuclei were counterstained with hematoxylin. As a control the antibodies were tested on well-known lymphoid tissue and specificity was tested by omitting the primary antibody.

Quantitative studies

Enumeration of the leucocytic subpopulations was performed in the urothelium, when it was not denuded, and in the lamina propria. Quantitative studies of CD4 and CD8 positives cells were carried out by counting in three to five random high-power fields with a X40 objective, and expressed as a ratio of $CD4^+/CD8^+$ cells respectively in the urothelium and the lamina propria. Analysis of the means ratio and its comparison in the different groups was achieved using the student Fischer test on small groups; a P value less then 0.05 was considered to be significant. The intensity of the lymphoid infiltrate, the number of $Leu7^+$ cells, the number of HLADR and/or Tac^+ cells, and the number of eosinophils were evaluated semiquantitatively (0 to +++).

Results

Group 1

The 27 specimens were obtained from patients treated for previously diagnosed TCC: 2 Tis, 19 Ta and 6 T1. On cryostat sections there was in every case an inflammatory infiltrate, the nature and intensity of which were not correlated with the initial histological staging of the TCC. The inflammatory cells were noted in the lamina propria and in the urothelium when the latter was not denuded. In 14 out of 27 cases there were few inflammatory cells in the lamina propria either in a random distribution, scattered diffusely, or in a band applied closely to the urothelium. In other instances, the inflammatory cells were more numerous (9 ++7/27 and 4 +++/27) and the infiltrate had spread to the muscle. This infiltrate was polymorphous; lymphoid follicles with germinal centres and epithelioid granulomata were not frequent, occuring respectively in only 7 and 6 out of 27 cases; neutrophil polymorphonuclear cells were unusual and plasmocytes were scattered. So the major cell populations of the infiltrate were lymphocytes, associated with macrophages and eosinophils. In the lamina propria and the urothelium the great majority (>90%) were $CD3^+$ lymphocytes. In comparing CD3 and CD4 antigen distribution it appeared that $CD4^+$ cells closely approximated the distribution of $CD3^+$ cells, although they were fewer; these $CD4^+$ cells were the predominant population present in the urothelium (Fig. 1a). All $CD4^+$ lympho-

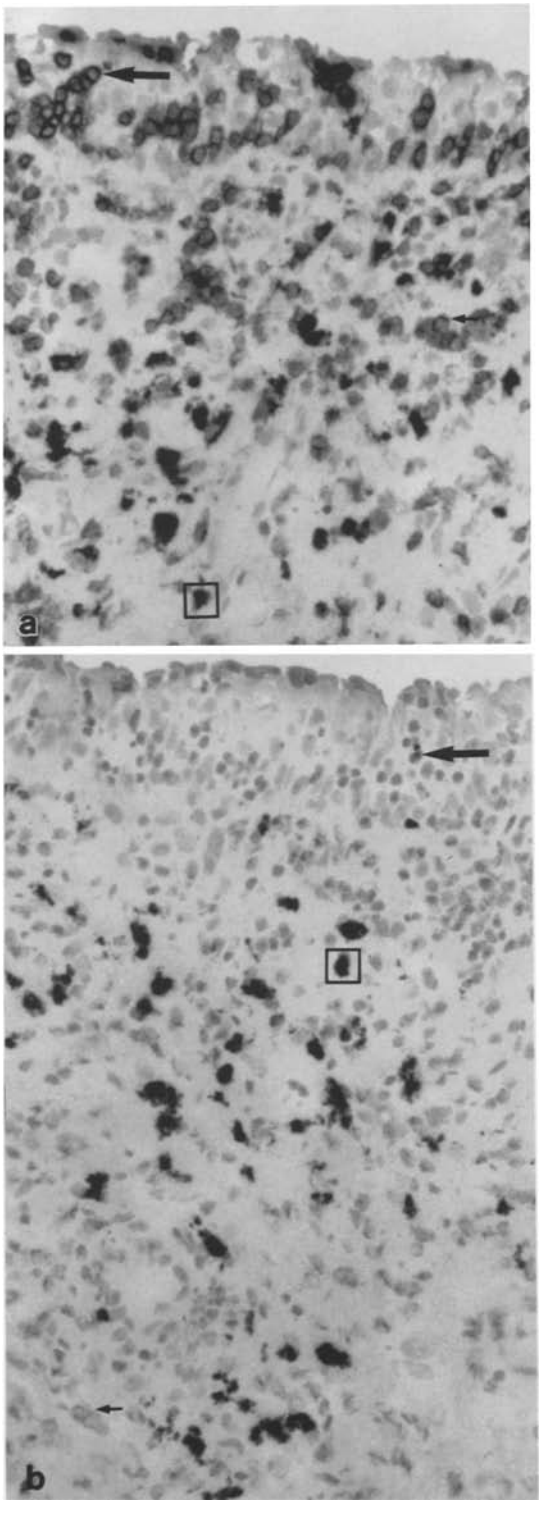

Fig. 1 a and b. Immunohistology of mucosal bladder tissue biopsies from a patient with superficial TCC treated with intravesical BCG. **a** Numerous $CD4^+$ T-lymphocytes in the urothelium (←) and in the lamina propria (↼), associated with polymorphonuclear eosinophils (□) [immunoperoxidase with leu3a (anti CD4) counterstained with hematoxylin, GX200]. **b** $CD8^+$ T-lymphocytes are scanty scattered in the urothelium (←) and in the lamina propria (↼); there were numerous eosinophils (□) [immunoperoxidase with leu2a (anti-CD8), counterstained with hematoxylin, GX200]

cytes were $TQ1^-$. The Leu3a antibody (anti-CD4) appeared to cross-ract with macrophages, but these were easily distinguished from T-cells by means of their dimensions.

$CD8^+$ cells were also present, scattered in the lamina propria and unusual in the urothelium (Fig. 1b). So the CD4/8 ratio was high in the lamina propria (mean = 6.80) as in the urothelium (mean = 4.87). Macrophages ($CD11^+$, $CD4^+$) were mainly seen in granulomata or, more unusually, scattered in the lamina propria. B cells ($CD22^+$) were seen only occasionally in the lamina propria; their numbers increased within recognizable lymphoid follicles; intraurothelial B cells were absent. HLA-DR and anti-Tac which were supposed to recognize activated T cells, were often expressed (20/27) on the surface of cells having the same distribution as $CD4^+$ cells; the intensity of the labelling was generally weak. It was interesting to note that the eosinophils were more numerous when HLA-DR and anti-Tac were positive. Leu 7^+ cells (Natural Killer Cells) were absent or uncommon in the lamina propria. In some cases the Leu7 antibody appeared to stain the urothelium in focal areas.

Group 2

In this group, histological studies found 7 superficial (Ta or T1) and 6 invasive (T2 or T3) tumors. On cryostat sections, whatever the histological staging, there was an important infiltrate with polymorphous cell population but predominantly lymphoid cells which expressed, as in group 1, the CD3 antigen. In the lamina propria the majority of the T-cells were $CD4^+$ cells. In the urothelium the majority of the T-cells expressed CD8.

Group 3

Here there was no malignant condition of the bladder and lymphocyte subpopulations were scanty, scattered singly in the lamina propria and in the urothelium. All these lymphocytes expressed CD3. Intraurothelial $CD3^+$ cells included a majority of $CD8^+$ cells, with only very few $CD4^+$ cells. In the lamina propria both CD4 and $CD8^+$ cells were found, the majority being $CD8^+$. Also scattered in the lamina propria were unusual macrophages ($CD11^+$ $CD4^+$) and $Leu7^+$ cells.

CD4/CD8 Ratio

Expression of this ratio in all group is summarized in Table 2.

Table 2. See text

	Group 1	Group 2	Group 3
CD4/8 in urothelium	6.80	0.48	0.27
CD4/8 in lamina propria	4.87	3.85	0.37

A reversal of the CD4/8 ratio was observed in the urothelium between on the one hand GI and on the other hand GII and III (significant difference, $P<0.01$) and in the lamina propria between GI and GIII (significant difference, $P<0.01$). There was no significant difference ($P>0.3$) between GI and GII for the CD4/8 ratio in the lamina propria.

Discussion

At the present time there are no data to support the hypothesis that a non-immunological mechanism (i.e. direct toxic effect or acute inflammation) mediates the anti-tumor activities of BCG. On the contrary many clinical and experimental observations are available to support the role of an immune response for antitumor activity. This evidence was discussed elsewhere [10, 11]. In brief it was based upon indirect data, showing a correlation between purified protein derivative skin test responsiveness and therapeutic efficacy of intravesical BCG [6], a correlation between the presence of granuloma in the mucosa of the bladder and a good response to therapy [6], and the presence of IL2 in urine specimen of BCG treated patients [1, 5]. In this respect Merguerian et al. [10] stated that BCG combined with IL2 administered intravesically was effective for the prophylactic treatment of bladder cancer, supporting the fact that exogenous IL2 stimulates the proliferation of antigen-activated T-cells. Experimentally Ratliff et al. [11] demonstrate that the inhibition of the growth of the intravesically implanted mouse bladder tumor MBT-2 was supported by a thymus dependant immune response. It has been demonstrated that in rats treated with intravesical BCG, the major cell population seen in the mononuclear infiltrate expressed a T phenotype with a preponderance of helper T-cells [12]. There were no data concerning the phenotype of mononuclear cells involving the human mucosa of the bladder after BCG therapy. For human studies we could only refer to those of Gardinier et al. [2] who demonstrate the presence of leukocytic cells in the normal human bladder mucosa; this reinforces the concept that this cell population could be regarded as another component of the mucosal-associated lymphoid tissue. It is important to note that in these normal bladder mucosa all lymphocytes located in the urothelium express CD8 and a majority of lymphocytes in the lamina propria also express CD8 [2]. These data are in agreement with the phenotype which we found in Group 3 in which all lymphocytes express CD3 and CD8 in the urothelium as in the Lamina propria; only a minor cell population was $CD3^+$ $CD4^+$ in the Lamina propria.

Our results clearly demonstrate that intravesical BCG therapy induces a mononuclear infiltrate which is different from that observed in the TCC of the bladder without BCG therapy (Group 2) and from the normal bladder (Group 3). These mononuclear cells both in the lamina propria as in the urothelium, were mainly T-cells expressing CD3 and in majority $CD4^+$ $TQ1^-$; this phenotype defined a subset supposed to contain the majority of helper function. In addition the cells were frequently activated expressing CD25 (Tac, Receptor – IL2) and HLA-DR. This is in agreement with the presence of IL2 in urine specimens of BCG-treated patient found by others authors [1, 5]. T-lymphocytes $CD4^+$ $CD25^+$ $HLA\text{-}DR^+$ are known to produce cytokines which seem to play a major role in the proliferation and/or the activation of the effector cells which support the antitumor activity of BCG.

The nature of these effector killer cells remains questionable and only a hypothesis could be made on the basis of the result of this study. Theoretically many cells could be putative killer cells like the cytotoxic T-cell, the Lymphokine activated killer cell (LAK cell), the natural killer cell (NK cell), the macrophage and the polymorphonuclear eosinophil. Ratliff et al. [11] state that NK cells were not the primary mediator of BCG which was in agreement with the paucity of NK cells which were found in Group 1. We can not rule out the importance of cytotoxic CD8 cells and macrophages, which are respectively activated by IL2 and BCG, but we have to point out that these subsets are weak in the BCG treated group. There is no mean to identify an eventual subset of LAK cells. The presence of eosinophils must be regarded with interest because the more the mononuclear cells are activated (i.e. Tac^+ and $HLA\text{-}DR^+$) the more numerous they become. Evidence suggests that cytolysis by eosinophils involves granule proteins released at the site of tumor target cells [14]. The hypothesis that $CD3^+$ $CD4^+$ cells produce lymphokines like interleukine 5 which stimulate the eosinophils must be considered.

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